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ORIGINAL ARTICLE

Rapid Detection of Rifampicin- and Isoniazid-Resistant *Mycobacterium tuberculosis* using TaqMan Allelic Discrimination

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Abstract
Objectives: Multidrug-resistant tuberculosis (MDR-TB) is a global problem that many countries are challenged with. Rapid and accurate detection of MDR-TB is critical for appropriate treatment and controlling of TB. The aim of the present study was to evaluate the TaqMan allelic discrimination without minor groove binder (MGB) as a rapid, efficient, and low-cost method for detection of drug resistant strains of *Mycobacterium tuberculosis*.
Methods: A total of 112 *M. tuberculosis* isolates from cases with diagnosed TB were subjected to drug susceptibility testing (DST), using the proportion method. Resistant isolates were tested for characterization of mutations in the *rpoB* and *KatG* genes by TaqMan genotyping.
Results: Of 112 *M. tuberculosis* isolates for which DST was performed, three, one, and two isolates were MDR, rifampin (RIF) resistant, and isoniazid (INH) resistant, respectively. According to the threshold cycle (Ct) and curve pattern of mutants, TaqMan probes detect all of the mutations in the analyzed genes (*katG* 315, AGC→ACC, *rpoB* 531, TCG→TTG, and *rpoB* 531, TCG→TGG).
Conclusion: The present study suggests that drug-resistant strains of *M. tuberculosis* can be detected by pattern's curve or Ct with TaqMan probes without MGB in real-time polymerase chain reaction (PCR).

1. Introduction

Multidrug-resistant tuberculosis (MDR-TB) is a global problem that many countries are challenged with. MDR-TB is caused by strains of *Mycobacterium tuberculosis* resistant to at least isoniazid (INH) and rifampin (RIF), the main first-line anti-TB drugs [1]. Rapid and accurate detection of MDR isolates is critical for appropriate treatment and controlling of TB [2–4]. The culture method is still the "gold standard" among other drug susceptibility

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testing methods of *M. tuberculosis* isolates. However, this method is time consuming and takes several weeks to be completed. In recent years, several molecular methods have been developed for detection of mutations in *rpo*B and *kat*G genes that cause resistance to INH and RIF [5–7]. Of these methods, Cepheid Xpert MTB/RIF kit and the line probe assays have been commercialized [8,9]. They give users the opportunity to detect the resistant mutants rapidly, but at very high cost. Therefore, it is not possible, particularly in developing countries, to apply these methods extensively. Thus, researches have been concentrated to introduce the in-house methods to reduce the cost.

TaqMan allelic discrimination is a new technique for detection of mutations. In this method, mutations even single nucleotide polymorphisms (SNP) are detected using TaqMan probe with Minor Groove Binder (MGB) and AmpliTaq Gold DNA polymerase. The efficiency of probe hybridization reduces whenever there are mismatches between a probe and allele. Moreover, AmpliTaq Gold DNA polymerase is more likely to displace the mismatched probe rather than cleave it to release reporter dye. Recently, Wada et al [10] used TaqMan minor groove binder (MGB) probes for detection of mutations in *rpoB*, *katG*, and *embB* genes of *M. tuberculosis*.

In this study, we designed the primers and probes for 81-bp hot spot of the *rpoB* gene and codon 315 of *katG* gene. Unlike most researches that used the TaqMan allelic discrimination, we did not use the MGB for designing of probes.

Our main purpose was to evaluate TaqMan allelic discrimination without minor groove binder (MGB) for rapid detection of drug-resistant strains of *M. tuberculosis*.

2. Materials and methods

2.1. Setting

This study was performed at the Tehran University of Medical Sciences. A total of 112 isolates of *M. tuberculosis* cultured from TB patients were included in this survey.

2.2. Drug susceptibility testing (DST)

Drug resistant isolates were identified among 112 isolates using proportion method on Lowenstein–Jensen (LJ) media [11].

2.3. DNA extraction

DNA was extracted from the bacterial colonies according to the method of van Soolingen et al [12]. A commercial DNA extraction kit (Invitek, Germany) was used for extraction of DNA from clinical specimens. The optical density of extracted DNA from standard strain and clinical specimens was determined at 260 nm using NanoDrop 1000 (Thermo Scientific, USA).

2.4. TaqMan analysis

TaqMan probes and primers for detection of mutations in *rpoB* and *katG* genes were designed using the Primer Express program (Applied Biosystems, Foster City, CA, USA) and were synthesized by Metabion (Martinsried, Germany). The primers and probes of *rpoB* gene were F: 5′-TCACACCGCAGACGTTGATC-3′, R: 5′-CGTAGTGCGACGGGTGC-3′, P1: 5′-FAM-CAGCT-GAGCCAATTCATGGACCAGA-BHQ-1-3′ (Tm = 67.2), and P2: 5′-VIC-CACAAGCGCCGACTGTCGGC-BHQ-1-3′ (Tm = 68.1). The primers and probe of *katG* gene were F: 5′-GGGCTTGGGCTGGAAGA-3′, R: 5′-GGAAACTGTTGTCCCATTTCG-3′, and P: 5′-FAM-ACGCGATCACCAGCGGCA-BHQ-1-3′ (Tm = 65.0).

The PCR reaction mixture was 1 × reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$], 2.5 mM of dNTPs, 2 U *Taq* DNA-polymerase (miTaq, Metabion, Martinsried, Germany), and 20 pmol each of primers in total volume of 50 μL. Amplification was performed in a Mastercycler Gradient (Eppendorf, Germany) using the following program: initial denaturation at 95°C for 10 minutes and 35 cycles of denaturation at 94°C for 25 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, and the final extension at 72°C for 5 minutes. After amplification, PCR products were sequenced.

The mixture of real-time PCR reaction was done using TaqMan Genotyping Master Mix (Applied Biosystems) and the mentioned primers and probes. The real-time PCR apparatus (Bioer, Hangzhou, and P.R., China) was programmed as follows: initial denaturation at 94°C for 10 minutes followed by 40 cycles of 94°C for 25 seconds, 60°C for 55 seconds. To obtain reproducibility of reaction, the amplification was run in triplicate on DNA obtained from H37Rv strain, mycobacterial isolates, and clinical samples. All samples were run with the DNA obtained from H37Rv strain. Human white blood cell DNA and beta actin gene were used as external and internal controls, respectively [13]. Diluted DNA of H37Rv was used for the evaluation of the sensitivity. Specificity of the reaction was evaluated on DNA from other mycobacteria and potentially pathogenic bacteria in the respiratory tract and human cells that was used in the previous study [14].

3. Results

Of 112 isolates, three, one, and two were MDR, RIF resistant and INH resistant, respectively. The results of sequencing of *rpo*B and *kat*G genes are shown in Table 1. Fig. 1 shows the analysis of DNA from mycobacteria with TaqMan probes. As described above, AmpliTaq Gold DNA polymerase could detect the mismatches between a probe and allele even SNP. According the figure, the pattern curve of H37Rv strain is different from the mutants. Moreover, the luminescence intensity

Table 1. Results of sequencing of 81-bp hot spot of the *rpoB* gene and codon 315 of the *katG* gene.

Phenotypic characteristic of resistant isolates	Genotypic characteristic of resistant isolates
Resistant to INH	*katG* 315, AGC→ACC
Resistant to RIF	*rpoB* 531, TCG→TTG
	rpoB 531, TCG→TGG

INH = isoniazid; RIF = rifampin.

or threshold cycle (Ct) was higher when mutations were present in the genes. The difference of curve patterns can differentiate the susceptible and resistance isolates. Therefore real-time PCR detected the all of the mutations in *rpoB* and *katG* genes.

With the exception of *M. tuberculosis* complex (*M. bovis, M. bovis, BCG, M. africanum*, and *M. microti),* no luminescence was found. The results of PCR in serial dilutions demonstrated high efficiency of the technique as it detected 10 pg of *M. tuberculosis* DNA.

4. Discussion

Real-time PCR system in combination with MGB probes has been applied to analyze single-nucleotide polymorphisms [10,15]. The MGB probes have been used in other studies for detection and distinction of one-base mismatches due to their high specificity and sensitivity [16], so it can be a powerful tool for control of drug-resistant *M. tuberculosis.* In this study, we developed a rapid, efficient, and low-cost real-time PCR-based system without MGB probes for detection of the mutations associated with INH and RIF resistance in *M. tuberculosis*.

Despite our probes detecting all mutations in the mentioned regions of the *katG* and *rpoB* genes as little as 3 hours from the preparation of DNAs from clinical samples, our method had limitation in use because it could not identify MDR and XDR strains that genetically belonged to the Harrlem3 and Harrlem4 clusters in spoligotyping. Interestingly, there were no mutations in the 81-bp hot spot of the *rpoB* gene and codon 315 of *katG* gene of these strains as determined by DNA sequencing (data not shown). Therefore, the result of this study gives evidence that a single molecular method for detection of drug-resistant *M. tuberculosis* is not sufficient. In comparison with the conventional and commercial methods (i.e., line probes or Cepheid Xpert MTB/RIF kit) for detection of MDR-TB, our in-house technique was less time- and fund-consuming, with costs five times cheaper than commercial kits.

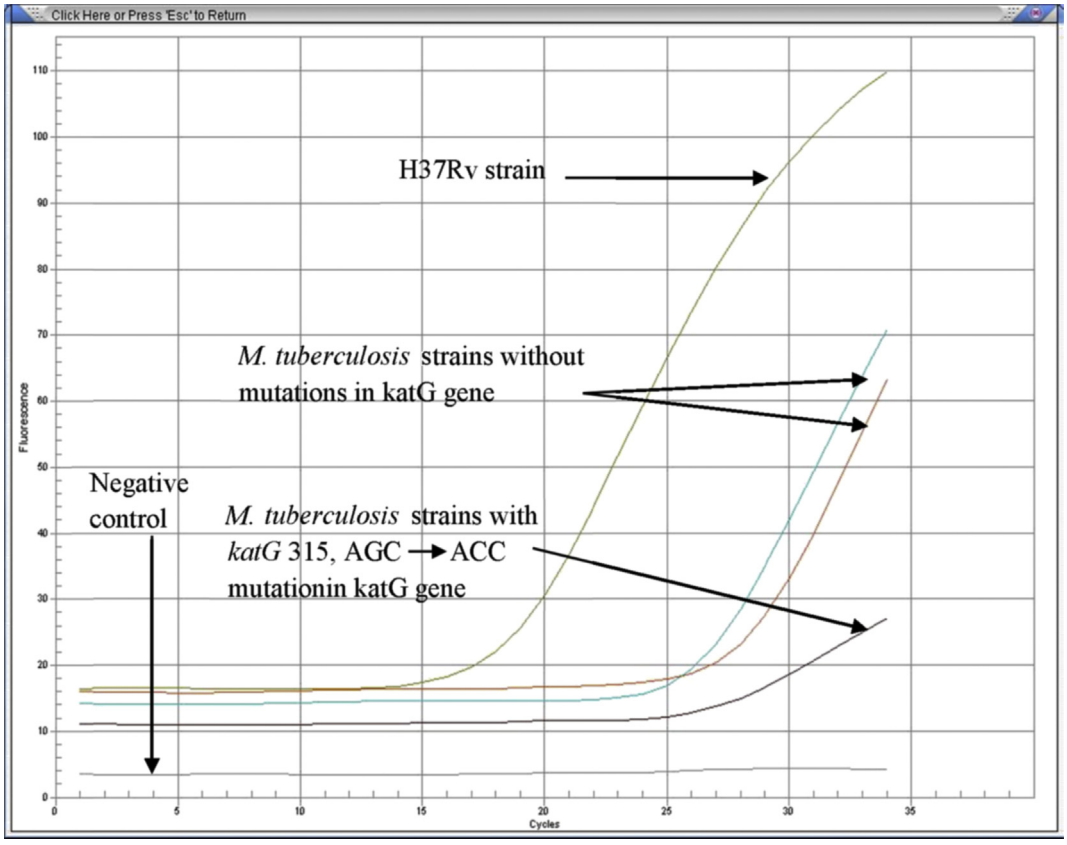

Figure 1. The curve patterns of H37Rv strain, negative control and *Mycobacterium tuberculosis* mutants with and without *katG* 315, AGC→ACC mutation.

Many studies used real-time PCR for detection of mutations in *rpoB* and *katG* genes [17−21] but, to our knowledge, this study is the first one that uses the TaqMan allelic discrimination without MGB. Our study suggests that drug-resistant *M. tuberculosis* can be detected by pattern's curve or Ct with TaqMan probes without MGB in real-time PCR. We evaluated our probes on limited resistant *M. tuberculosis* isolates (*katG* 315, AGC→ACC, *rpoB* 531, TCG→TTG and *rpoB* 531, TCG→TGG), and the mentioned mutations are the common mutations that cause RIF- and INH-resistant strains. However, for more confirmation, evaluation of other mutations in the 81-bp hot spot of the *rpoB* gene as well as more drug-resistant strains is needed.

Conflicts of interest

All authors have no conflicts of interest to declare.

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